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REGULAR ARTICLE

# Density of human bone marrow stromal cells regulates commitment to vascular lineages

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**Abstract** Mechanisms underlying the vascular differentiation of human bone marrow stromal cells (HBMSCs) and their contribution to neovascularisation are poorly understood. We report the essential role of cell density-induced signals in directing HBMSCs along endothelial or smooth muscle lineages. Plating HBMSCs at high density rapidly induced Notch signaling, which initiated HBMSC commitment to a vascular progenitor cell population expressing markers for both vascular lineages. Notch also induced VEGF-A, which inhibited vascular smooth muscle commitment while consolidating differentiation to endothelial cells with cobblestone morphology and characteristic endothelial markers and functions. These mechanisms can be exploited therapeutically to regulate HBMSCs during neovascularisation.

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## Introduction

**Abbreviations:**  $\alpha$ SMA, smooth muscle alpha actin; DAPT, *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *t*-butyl ester; DMSO, dimethyl sulfoxide; ECs, endothelial cells; EDTA, ethylenediaminetetraacetic acid; HES-1, hairy enhancer of split-1; HUVECs, human umbilical vein endothelial cells; LDL, low density lipoprotein; HBMSCs, human bone marrow stromal cells; PECAM-1, platelet endothelial cell adhesion molecule-1 (CD31); SM-MHC-1, vascular smooth muscle myosin heavy chain 1; VCAM-1, vascular cell adhesion molecule; VE-cadherin, vascular endothelial cadherin; VEGF, vascular endothelial growth factor; VEGFR1, vascular endothelial growth factor receptor 1; VEGF-I, VEGF neutralization antibody; VEGFR-I, VEGFR signaling inhibitor; vSMC, vascular smooth muscle cell; vWF, Von Willebrand factor.

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Human bone marrow stromal cells (HBMSCs) have therapeutic potential in cell transplantation and tissue engineering. In addition to their anti-inflammatory and immunomodulatory characteristics (Lee et al., 2009a; Casiraghi et al., 2010), they have the capacity to differentiate directly along skeletal and other mesenchymal lineages (Pittenger et al., 1999; Augello and De Bari, 2010). A major challenge now is to define the mechanisms that regulate their differentiation along vascular endothelial cell (EC) and smooth muscle cell (vSMC) lineages.

Cultured HBMSCs typically possess some characteristics of synthetic proliferative vSMCs. In particular, they express the early contractile marker smooth muscle alpha actin ( $\alpha$ SMA), as well as calponin and smoothelin-B (Ball et al., 2004). Stimuli shown to induce the differentiation of HBMSCs towards vSMCs include mechanical strain (Kurpinski et al., 2006), inhibition of DNA methylation (Wakitani et al., 1995), and transforming growth factor (TGF) $\beta$  and Notch signaling (Kurpinski et al., 2010). Other studies

have indicated that HBMSCs have the potential to differentiate towards EC lineages (Al-Khalidi et al., 2003; Bai et al., 2009; Chen et al., 2009; Chung et al., 2009; Lozito et al., 2009; Ohata et al., 2009; Oswald et al., 2004; Siekmann et al., 2008; Wu et al., 2005a; Xu et al., 2009; Yue et al., 2008; Zhang et al., 2008). In many of these studies, HBMSCs exposed over days to exogenous vascular endothelial growth factor (VEGF-A) showed evidence of differentiation towards ECs. However, HBMSCs do not always express vascular endothelial growth factor receptors (VEGFRs) (Chen et al., 2009; Oswald et al., 2004; Ball et al., 2007). Notch signaling has also been shown to direct HBMSCs towards an EC fate when activated experimentally using recombinant ligands, chemical exposure, or expression constructs (Ohata et al., 2009; Siekmann et al., 2008; Xu et al., 2009; Zhang et al., 2008).

Notch and VEGF signaling coordinate endothelial cell activity during vascular development, arterio-venous specification, vessel branching and endothelial tip cell formation (Gridley, 2007; Lawson et al., 2001; Boulton et al., 2008; Roukens et al., 2010; Jakobsson et al., 2010). We have investigated how these signaling mechanisms also direct the commitment of HBMSCs toward vascular ECs or vSMCs. We report that plating HBMSCs at high density rapidly stimulates Notch signaling, which induces vascular progenitor cells with the capacity to give rise to either EC or vSMC lineages. VEGF-A is not essential for the initiation of HBMSC differentiation to ECs but regulates the fate decision between EC and vSMC lineages. These mechanistic insights may be exploited in therapeutic applications of HBMSCs.

## Results

### HBMSCs plated and cultured at high density differentiated toward endothelial cells

Cell density can regulate the morphology and differentiation of HBMSCs (Ball et al., 2004; McBeath et al., 2004). Using HBMSCs which could be induced to differentiate toward adipocytes, osteocytes, or chondrocytes (Supplementary Fig. S1), we studied the impact of plating HBMSCs at high cell density on their differentiation toward ECs, monitoring morphology (Fig. 1A), specific functional properties (Fig. 1B), and expression of a panel of characteristic endothelial markers (Figs. 1C and D).

HBMSCs plated at high density and cultured for 14 days (Fig. 1AIII) adopted a cobblestone-like morphology comparable to that of human umbilical vein endothelial cells (HUVECs) (Fig. 1AIV), whereas HBMSCs cultured under standard conditions for 48 h (Fig. 1AI), or at low cell density for 14 days (Fig. 1AII), maintained a spindle-shaped morphology.

HBMSCs plated at high density and cultured for 28 days displayed low density lipoprotein (LDL) uptake in approximately 53% of cells analysed (Fig. 1BIII), and VCAM-1 was also induced in response to  $\text{TNF}\alpha$  (Fig. 1BVI). In contrast, HBMSCs cultured under standard conditions for 48 h did not display LDL uptake (Fig. 1BII) or VCAM-1 induction in response to  $\text{TNF}\alpha$  (Fig. 1BV). As expected, HUVECs as positive control cells exhibited EC functional characteristics of LDL uptake (Fig. 1BI) and induction of VCAM-1 in response to  $\text{TNF}\alpha$  (Fig. 1BIV).

The distribution of the EC markers VE-cadherin, PECAM-1, vWF, and VEGFR1 in HBMSCs was examined by immunofluorescence microscopy. HBMSCs plated at high density and cultured for 14 or 28 days displayed prominent EC marker immunostaining (Figs. 1CIII and VI, DIII and VI). In contrast, HBMSCs cultured under standard conditions for 48 h displayed little or no immunofluorescence for the EC markers examined (Figs. 1CII and V, DII and V). As expected, HUVECs as positive control cells expressed the characteristic markers VE-cadherin (Fig. 1CI), PECAM-1 (Fig. 1CIV), vWF (Fig. 1DI), and VEGFR1 (Fig. 1DIV).

### High density HBMSCs expressed endothelial and smooth muscle cell markers by 24 h

As plating at high cell density for up to 28 days induced HBMSCs to adopt an endothelial phenotype (Fig. 1), we further investigated the time scale of these cellular changes by examining the expression of vascular cell markers following HBMSC culture at high density for 24 h and 3 days. The expression of EC (vWF, VEGFR1 and PECAM-1) (Figs. 2A–C) and vSMC ( $\alpha$ SMA, calponin, smoothelin-B, and SM-MHC-1) (Figs. 2D–G) markers were analysed by immunoblot analysis. Compared to HBMSCs cultured at low density for 24 h (lane 1), culturing HBMSCs at high density (lane 2) not only induced the expression of the EC markers vWF (Fig. 2A), VEGFR1 (Fig. 2B), and PECAM-1 (Fig. 2C), but markedly up-regulated the expression of vSMC markers  $\alpha$ -SMA (Fig. 2D), calponin (Fig. 2E), smoothelin-B (Fig. 2F), and SM-MHC-1 (Fig. 2G). To determine whether individual HBMSCs were expressing both EC and vSMC markers, double-labeling immunofluorescence analysis was conducted (Fig. 2H). This analysis clearly demonstrated that individual HBMSCs were coexpressing EC and vSMC markers following culture at high cell density for 24 h. Thus, both EC and vSMC markers were enhanced within 24 h of plating HBMSCs at high density, indicating a vascular progenitor phenotype with potential to form ECs or vSMCs.

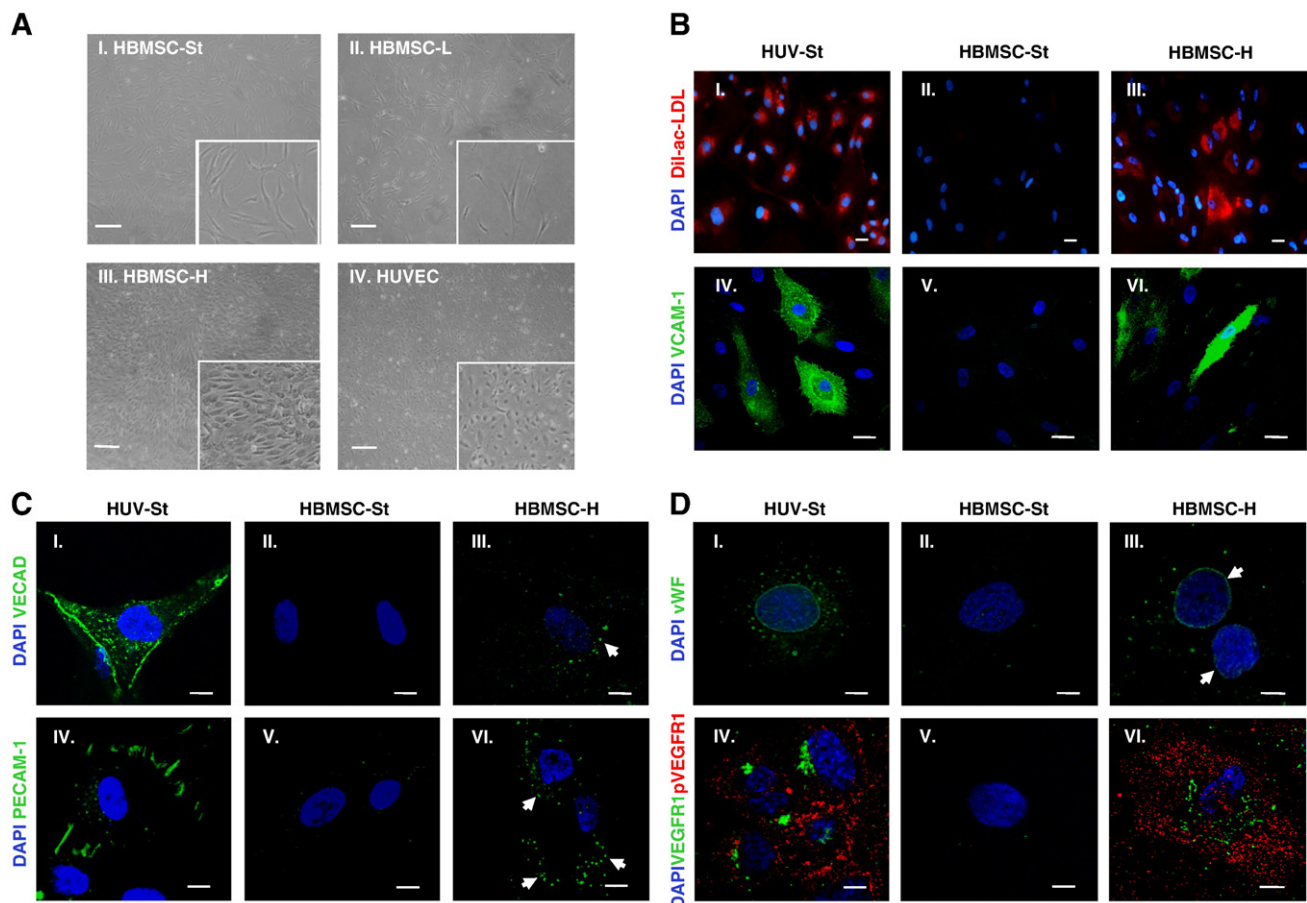
### High density HBMSCs enhanced endothelial markers but down-regulated smooth muscle cell markers by 3 days

HBMSC expression of vascular markers was further examined after 3 days. Compared to HBMSCs cultured at low density, the level of VEGF-A secreted by HBMSCs at high cell density, normalised against corresponding  $\beta$ -actin levels, was significantly increased over a 3-day period (Fig. 3A). This enhanced VEGF-A secretion also coincided with a significant increase in the expression of EC markers (Figs. 3B and C); however, in comparison, expression of vSMC markers SM-MHC-1 (Fig. 3D) and smoothelin-B (Fig. 3E) dramatically decreased by 3 days. Thus, sustained culture of HBMSCs plated at high density promoted EC differentiation but inhibited vSMC differentiation.

### Notch signaling induced vascular progenitor cells and VEGF-A secretion

Notch signaling is known to regulate vascular remodeling (Gridley, 2007). To investigate whether signaling through Notch receptors controlled the density-dependent differentiation of HBMSCs toward vascular phenotypes, Notch signaling molecules were examined up to 3 days after plating HBMSCs at high density (Fig. 4). The Notch transcription factor HES-1 was not detected in HBMSCs cultured at low cell density, but was rapidly induced within 24 h of plating HBMSCs at high density, and levels remained high during 3 days culture (Fig. 4A). To investigate whether Notch signaling may contribute to inducing vascular progenitor cells within 24 h, HBMSCs plated at low density or high density were immunostained for Notch receptors 1–4. Only the high density HBMSCs strongly expressed Notch receptors 1, 2, and 3, (Supplementary Fig. S2), while Notch receptor 4 was not detected (not shown). This result was confirmed by qPCR and immunoblot analysis (not shown).

DAPT is a chemical inhibitor of Notch signaling that inhibits the transcription of target genes, including HES-1 (Boulton et al., 2008) (Supplementary Fig. S3A). HBMSCs plated at high density were therefore treated with DAPT for 24 h, and then the expression of markers for EC and vSMC were determined by immunoblot analysis (Figs. 4B–D). HBMSCs plated at high density and cultured in the presence of DAPT showed a significant decrease in VEGFR1 and vWF (Figs. 4B and C) and SM-MHC-1 (Fig. 4D) compared to DMSO-treated



**Figure 1** HBMSCs plated and cultured at high density differentiated toward functional ECs. To determine whether HBMSCs plated and cultured at high density differentiated toward EC over time, their morphology, EC functional properties, and expression of EC markers were determined. (A) Representative phase contrast images of (I) HBMSCs under standard culture conditions (MSC-St) for 48 h, (II) HBMSCs cultured at low density (HBMSC-L) for 14 days, or (III) HBMSCs cultured at high density (HBMSC-H) for 14 days. (IV) HUVECs under standard culture conditions were used as a positive cellular control. For culture conditions, see Materials and methods. Images were obtained using an Olympus (CK X41) microscope (4x objective). Inserts represent an enlarged region of each image. Scale bars=200  $\mu$ m. (B) (I–III) Immunofluorescence analysis showing Dil-Ac-LDL uptake in (I) HUVECs, (II) HBMSCs under standard culture conditions (HBMSC-St) for 48 h, and (III) HBMSCs cultured at high density (HBMSC-H) for 28 days. Dil-Ac-LDL uptake (red), DAPI (blue). Images obtained using an Olympus upright widefield fluorescence (BX51) microscope (20x objective). Scale bars=20  $\mu$ m. (IV–VI) Immunofluorescence analysis of VCAM-1 in cells stimulated with 10  $\mu$ g/ml TNF $\alpha$  for 24 h. (IV) HUVECs under standard culture conditions for 48 h, (V) HBMSCs under standard culture conditions (HBMSC-St) for 48 h, and (VI) HBMSCs at high density (HBMSC-H) for 28 days. VCAM-1 (green), DAPI (blue). Images were obtained using a Nikon C1 upright confocal microscope (60x objective). Scale bars=20  $\mu$ m. (C,D) Immunofluorescence analysis of VE-cadherin, PECAM-1, vWF, and VEGFR1 expression in (I, IV) HUVECs under standard culture conditions for 48 h, (II, V) HBMSCs under standard culture conditions for 48 h, or (III, VI) HBMSCs cultured at high density (HBMSC-H) for (C) 28 days or (D) 14 days. VE-cadherin, PECAM-1, vWF, and VEGFR1 (green), phosphorylated VEGFR1 (red), DAPI (blue). Images were obtained using a Nikon C1 upright confocal microscope (60x objective) (C, I–VI; and D, IV–VI) or using an Olympus IX71 Deltavision microscope (40x objective) (D, I–III). Scale bars=10  $\mu$ m. White arrows highlight specific regions of localised immunoreactivity. Representative images from two independent experiments.

control HBMSCs. In addition, the level of VEGF-A secreted by DAPT-treated HBMSCs at high density for 24 h, normalised against corresponding  $\beta$ -actin levels, was also significantly inhibited (Fig. 4E). Thus, Notch signaling enhanced VEGF-A secretion and induced a vascular progenitor cell state.

To examine whether Notch receptors 1–3 were involved in inducing vascular progenitor cells and stimulating VEGF-A secretion, siRNA knockdown was performed. The efficacy of each siRNA knockdown compared to scrambled siRNAs was confirmed by immunoblot analysis (Supplementary Fig. S3B). The specificity of each siRNA Notch receptor knockdown was confirmed by reprobing

the blots for a nontarget Notch receptor, which revealed unchanged protein levels in each case (Supplementary Fig. S3B). Knockdown of Notch receptors was therefore used to investigate which Notch receptors were involved in regulating the expression of vascular markers in HBMSCs plated at high density for 24 h (Figs. 4F–I). Knockdown of Notch receptor 1, 2, or 3 demonstrated a significant decrease both in VEGFR1 expression (Fig. 4F) and in VEGF-A secretion (Fig. 4I). Knockdown of Notch receptor 1 or 2 significantly inhibited vWF expression (Fig. 4G, lanes 2 and 3), while knockdown of Notch receptors 1 or 3 significantly inhibited SM-MHC-1 expression (Fig. 4H, lanes 2 and 4).



Thus, Notch signaling activated by plating HBMSCs at high cell density is required for induction of vascular progenitor cells and commitment toward ECs.

### Notch activation stimulated HBMSCs at low density to express EC markers

We examined whether activation of Notch signaling could induce the vascular potential of HBMSCs plated and cultured at low cell density. Notch signaling can be activated by treating cells with cation chelators, which cause the rapid shedding of the Notch extracellular domain, thereby increasing Notch receptor 1 intranuclear staining and transcription of a nuclear mediator of Notch signaling (Rand et al., 2000; Aster et al., 1999; Rand et al., 1997). HBMSCs plated at high density and cultured for 24 h in the presence of 5 mM EDTA displayed an increase in HES1, demonstrating the efficacy of EDTA treatment (Supplementary Fig. S3C). While neither VEGFR1 nor vWF were detected when HBMSCs were plated at low cell density and cultured for 24 h in the absence of EDTA (Figs. 5A and B, lanes 1), HBMSCs cultured at low cell density in the presence of EDTA expressed both VEGFR1 (Fig. 5A, lane 2) and vWF (Fig. 5B, lane 2). Thus, activation of Notch signaling by EDTA exposure induced the expression of EC markers that would otherwise only be expressed by HBMSCs plated at high density.

To confirm that HBMSCs plated at low density and cultured for 24 h could be induced to up-regulate expression of vWF by Notch activation, HBMSCs at low cell density were exposed to the Notch ligand Jagged-1 (Fig. 5C). Immunoblot analysis revealed that, compared to unstimulated HBMSCs (Fig. 5C, lane 1), exposure to

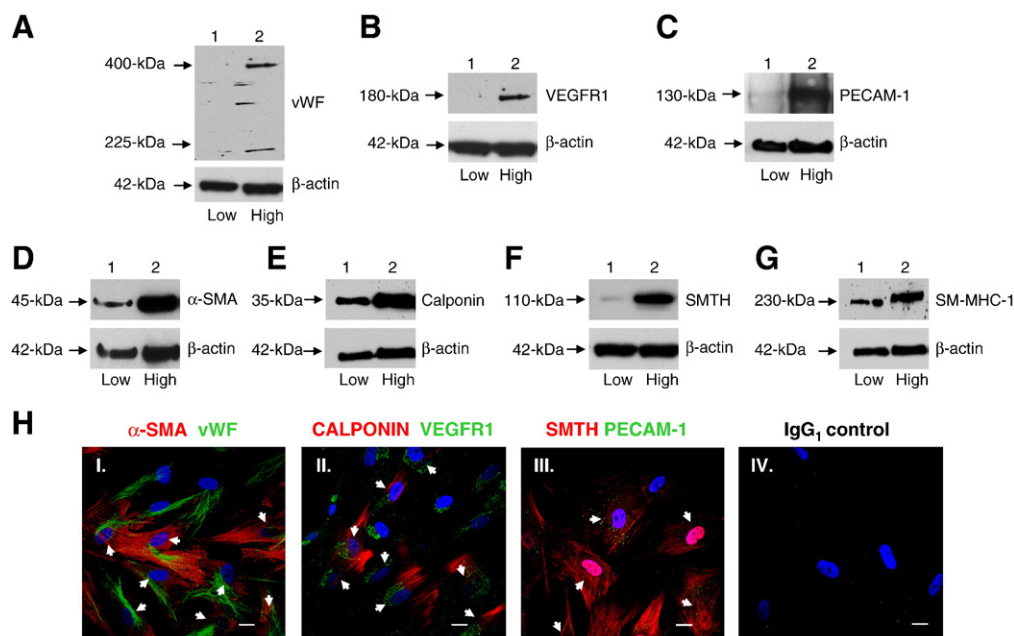
Jagged-1 induced HBMSCs at low cell density to enhance vWF expression (Fig. 5C, lane 2).

Thus, Notch signaling was sufficient to stimulate EC marker expression in HBMSCs at low cell density.

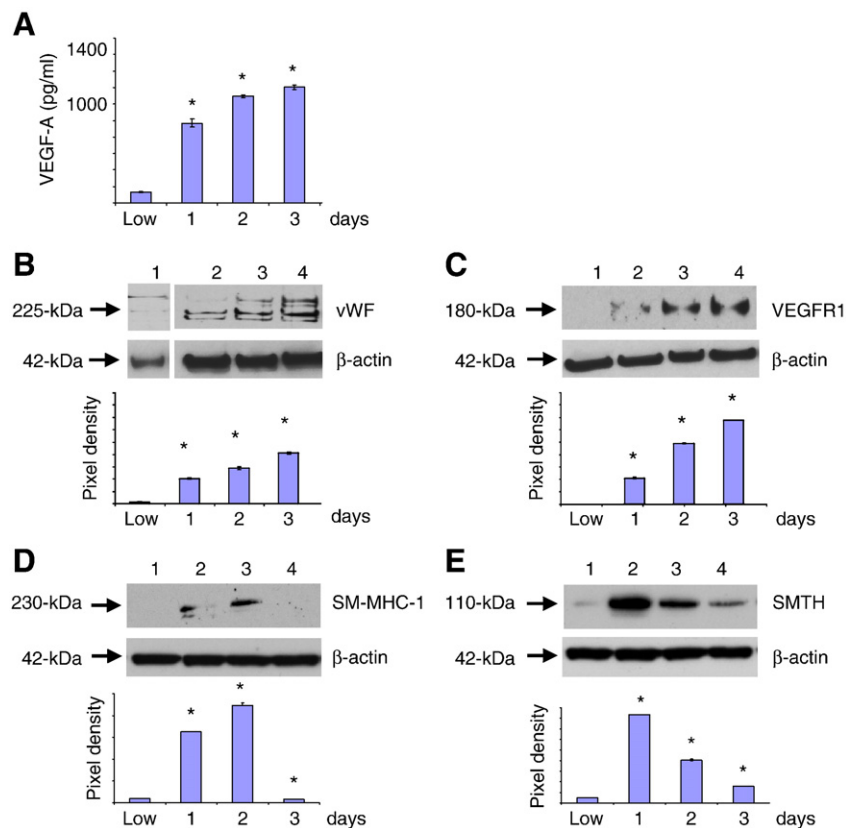
### VEGF-A promoted EC differentiation but inhibited vSMC differentiation

Since HBMSCs plated at high density for 24 h significantly increased their VEGF-A secretion, which was Notch dependent (Fig. 4E), experiments were conducted to determine if Notch-induced VEGF-A inhibited differentiation toward vSMCs. When HBMSCs that had been plated at high cell density and cultured for 24 h in the presence of a VEGF neutralisation antibody (designated VEGF-I) (Supplementary Fig. S3D), there was a significant up-regulation of SM-MHC-1 (Fig. 6A) and desmin expression (data not shown). To confirm that VEGF-A suppressed SM-MHC-1 expression, VEGF siRNA knockdown was performed (Supplementary Figs. S3E and F). VEGF-A knockdown was shown significantly to up-regulate SM-MHC-1 (Fig. 6B), indicating that VEGF-A was inhibiting expression of this definitive vSMC contractile marker.

VEGF-A has been implicated in directing HBMSC differentiation toward ECs (see Introduction). However, exposure to exogenous VEGF-A for 24 h did not initiate HBMSC commitment to ECs (Supplementary Figs. S4A–C). To investigate whether, over a longer time frame, VEGF-A influenced HBMSC commitment to ECs, HBMSCs were plated at high density and cultured for 14 days in the absence or presence of VEGF-I, or a chemical inhibitor of VEGF signaling (designated VEGFR-I) (Supplementary Fig. S3G). In the presence of



**Figure 2** HBMSCs plated at high density and cultured for 24 h expressed EC and vSMC markers. HBMSCs plated and cultured at high density for 24 h were examined for the expression of vascular cell markers. (A–G) Immunoblot analysis of HBMSCs cultured at high density for 24 h to determine the expression of markers for EC and vSMC. (A) vWF, (B) VEGFR1, (C) PECAM-1, (D) αSMA, (E) calponin, (F) smoothelin-B (SMTH), and (G) SM-MHC-1 in HBMSCs cultured at low density for 24 h (lane 1) or at high density for 24 h (lane 2). Membranes were reprobed with β-actin as loading controls. PECAM-1 was immunoprecipitated prior to immunoblotting and equal volumes of protein lysate were immunoblotted for β-actin as loading controls. Representatives of two independent experiments are shown for each analysis. (H) Double-labeling immunofluorescence analysis to detect individual HBMSCs coexpressing EC and vSMC markers following culture at high cell density for 24 h. vSMC markers (I) αSMA, (II) calponin, and (III) smoothelin-B (red); EC markers (I) vWF, (II) VEGFR1, and (III) PECAM-1 (green); DAPI (blue). The isotype control IgG<sub>1</sub> is also depicted. Images were obtained using a Nikon C1 upright confocal microscope (60x objective). Scale bars represent 20 μm. White arrows highlight individual HBMSCs coexpressing EC and vSMC markers. Representative images obtained from two independent experiments are shown.



**Figure 3** High density HBMSCs expressed increased endothelial cell markers but reduced smooth muscle cell markers, by 3 days. The levels of vascular cell markers were determined in HBMSCs plated and cultured at high density for 3 days. (A) ELISA assay of VEGF-A derived from medium taken from HBMSCs cultured at low density for 24 h, or at high cell density up to 3 days, normalised against corresponding  $\beta$ -actin levels. (B–E) Immunoblot analysis of (B) vWF, (C) VEGFR1, (D) SM-MHC-1, and (E) smoothelin-B (SMTH) in HBMSCs cultured at low density (HBMSC-L) for 24 h or at high cell density up to 3 days. Pixel density was normalised to  $\beta$ -actin and plotted as bar graphs. \*  $P < 0.05$  compared to HBMSCs cultured after plating at low cell density.

VEGF-I or VEGFR-I, HBMSCs showed a significant reduction in VEGFR1 expression (Fig. 6C) and VE-cadherin expression (Fig. 6D). Thus, although VEGF-A is not sufficient to initiate HBMSC differentiation to ECs, it enhances EC differentiation in sustained high cell density cultures.

To confirm that VEGF-A stimulated VEGFR1 expression over 14 days, HBMSCs plated at high density were exposed to recombinant VEGF-A. Following culture of HBMSCs at high density in the presence of VEGF-A, VEGFR1 transcript (Fig. 6E) and protein expression (Fig. 6F) were significantly enhanced over this time frame. Thus, Notch-induced VEGF-A supports EC differentiation in sustained high cell density cultures.

### Tubule formation by HBMSCs precultured at high density

HBMSCs that had been precultured for up to 28 days at high cell density were cultured on Matrigel and exposed to chick chorioallantoic membranes (CAM). Both control and high density cultured HBMSCs readily formed widespread tubule networks on Matrigel, but only high density cultured HBMSCs expressed EC markers (Fig. 7A).

While exposure to the CAM induced control HBMSCs to express EC markers (vWF, VE-cadherin, PECAM-1, and VEGFR2), in comparison, HBMSCs cultured at high density produced more abundant EC marker expression, formed more robust tubules (Fig. 7B), and were found to induce significantly larger blood vessels in the CAM (Fig. 7C).

We also evaluated the ability for HBMSCs precultured at high density to retain their EC phenotype when subcultured at low cell density. HBMSCs cultured at high density for 28 days were trypsinised and subcultured at low cell density for 7 days, and then their level of VEGFR1 and vWF expression was determined by immunoblot analysis (Supplementary Fig. S4D). Compared to HBMSCs cultured at high density for 28 days, those subsequently subcultured at low density for 7 days expressed only slightly lower levels of VEGFR1 and vWF, suggesting that the EC phenotype was maintained.

Together, these data show that coordinated Notch and VEGF-A signals direct the differentiation of HBMSCs toward ECs.

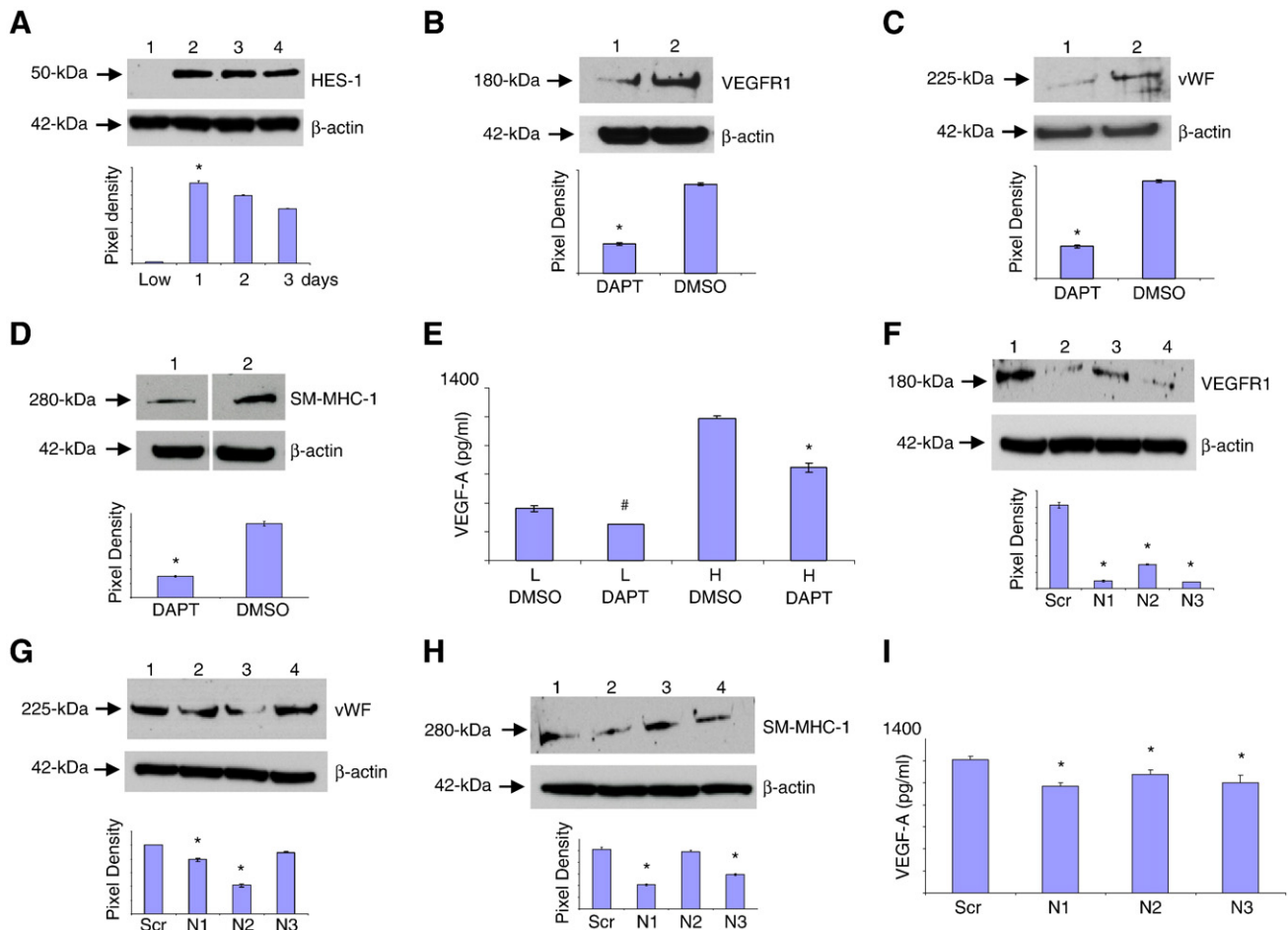
### Discussion

Defining the mechanisms that regulate HBMSC differentiation along vascular cell lineages will advance opportunities to exploit them in cell-based and neovascularisation therapies. While HBMSC differentiation toward vSMCs involves mechanical strain (Kurpinski et al., 2006), DNA methylation changes (Wakitani et al., 1995), and TGF $\beta$  and Notch signaling (Kurpinski et al., 2010), mechanisms underlying the differentiation of HBMSCs to ECs remain incompletely understood but largely attributed to VEGF-A. In this study, the inductive effect of cell contact has been identified as a potent trigger to vascular differentiation (Fig. 8). Cell contact-activated Notch signaling initially induced vascular progenitor cells with EC and vSMC characteristics. Subsequent differentiation toward ECs, but

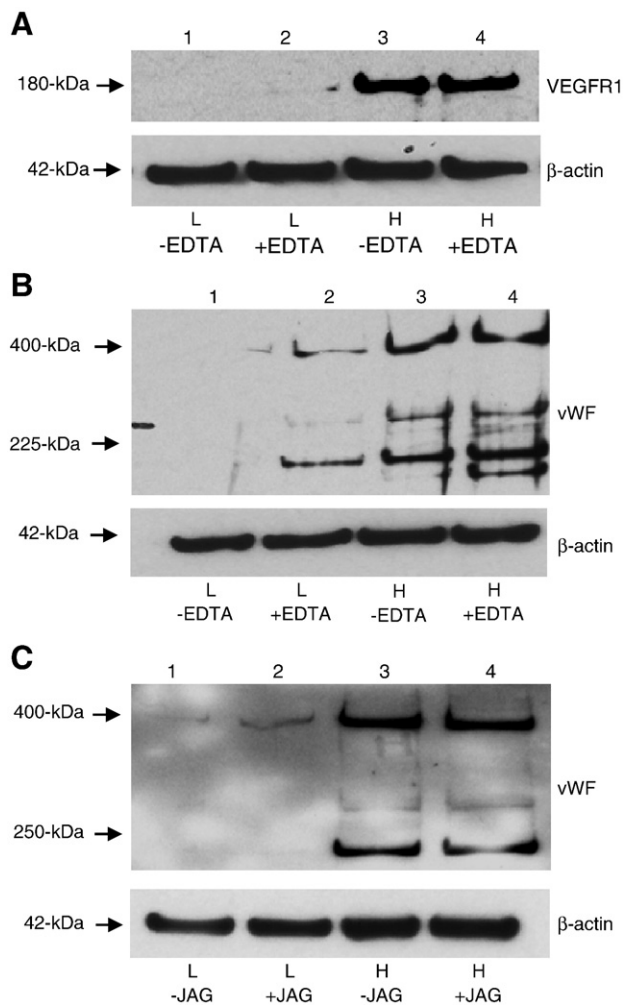
not vSMCs, was regulated by Notch-induced VEGF-A. Thus, coordinated Notch and VEGF-A signaling directs HBMSCs toward ECs.

Notch receptors are critical for vascular development and function (Gridley, 2007; Lawson et al., 2001; Boulton et al., 2008; Roukens et al., 2010; Jakobsson et al., 2010). In this study, Notch receptors 1–3 were directly implicated in the induction of vascular progenitor status in the HBMSCs cultured at high density, and thus contribute to vascular commitment. Although Notch 4 can be expressed in vascular cells (Wu et al., 2005b), we did not detect Notch 4 either at 24 h after plating at high density or by 28 days (not shown). Thus, Notch 4 appears not to be necessary for the differentiation of HBMSCs to ECs.

Density-dependent Notch signaling rapidly induced a vascular progenitor cell state characterised by expression of EC and vSMC markers. Since VEGFR1 is not endothelial-specific, despite being a major EC product (Supplementary Fig. S5), several other endothelial markers were utilized including VEGF-A, VE-cadherin, PECAM-1, and vWF. We found that VEGF-A was strongly induced by Notch, which in turn stimulated commitment of progenitor HBMSCs along the EC lineage. Interestingly, exposure to exogenous VEGF-A at standard density over 24 h was not sufficient to induce HBMSCs to express EC markers (Supplementary Fig. S4). Therefore, VEGF-A is not sufficient to initiate HBMSC differentiation to ECs, although it supports differentiation in sustained high density cultures. *In vivo* studies have documented how VEGF can disrupt vSMC function, ablating



**Figure 4** Notch signaling stimulated the formation of the vascular progenitor cell and VEGF-A secretion. The role of Notch signaling in regulating the expression of vascular cell markers in HBMSCs at high cell density was determined. (A) Immunoblot analysis of HES-1 in HBMSCs cultured at low density (lane 1) for 24 h or at high density up to 3 days (lanes 2–4). (B–D) To investigate whether Notch signaling regulated the expression of vascular cell markers and VEGF-A, their protein levels were determined following inhibition of Notch signaling by DAPT treatment (50  $\mu$ M), with DMSO used as a diluent control. Immunoblot analysis of (B) VEGFR1, (C) vWF, or (D) SM-MHC-1 from high density HBMSCs cultured in the presence of DAPT (lanes 1) or DMSO (lanes 2). Pixel density was normalised to  $\beta$ -actin and plotted as bar graphs. \*  $P < 0.05$  compared to HBMSCs plated at high density in the presence of DMSO only. (E) ELISA assay of VEGF-A derived from the medium taken from HBMSCs cultured at low cell density (L) or high cell density (H) in the presence of DMSO as a diluent control, or DAPT, normalised against corresponding  $\beta$ -actin levels. \*  $P < 0.05$  compared to HBMSCs cultured after plating at high cell density in the presence of DMSO only. (F–I) To confirm that Notch signaling induced vascular marker expression and VEGF-A secretion, siRNA knockdown of Notch receptors 1, 2, or 3 (N1–N3) was performed. Immunoblot analysis of (F) VEGFR1, (G) vWF, and (H) SM-MHC-1 from HBMSCs cultured for 24 h after plating at high cell density, following transfection with scrambled (Scr) controls (lane 1) or siRNAs for Notch receptors 1, 2, and 3 (lanes 2–4). Membranes were reprobed with  $\beta$ -actin as loading controls. Pixel density was normalised to  $\beta$ -actin and plotted as a bar graph. \*  $P < 0.05$  compared to scrambled control-transfected HBMSCs. Data are representative of two independent experiments. (I) ELISA assay of VEGF-A derived from medium taken from HBMSCs cultured at high cell density following Notch receptor siRNA knockdown, normalised against corresponding  $\beta$ -actin levels. \*  $P < 0.05$  and #  $P < 0.1$  compared to scrambled control HBMSCs.



**Figure 5** Notch activation stimulated HBMSCs at low density to express EC markers. To determine whether experimental activation of Notch could induce vWF and VEGFR1 protein expression in HBMSCs plated at low density and cultured for 24 h, HBMSCs were treated with Notch activators. (A,B) Immunoblot analysis of (A) VEGFR1 or (B) vWF protein expression from HBMSCs cultured after plating at low density (L) (lanes 1 and 2), or high cell density (H) (lanes 3 and 4), in the absence (–) (lanes 1 and 3) or presence (+) (lanes 2 and 4) of 5 mM EDTA. (C) To verify that HBMSCs cultured at low density could be induced to up-regulate vWF protein expression by activation of Notch signaling, Notch was activated using 5 μM Jagged-1. Immunoblot analysis of vWF protein expression in HBMSCs cultured after plating at low density (lanes 1 and 2) or high cell density (lanes 3 and 4) in the absence (–) (lanes 1 and 3) or presence (+) (lanes 2 and 4) of 5 μM Jagged-1. Membranes were reprobed with β-actin as loading controls. Data are representative of two independent experiments.

pericyte coverage and causing vessel destabilisation (Greenberg et al., 2008). Thus, the Notch-induced VEGF-A may have inhibited HBMSC differentiation to vSMCs while promoting HBMSC differentiation to ECs, and tubule formation.

We investigated several functional EC features of the high density HBMSCs. Uptake of LDL is a characteristic function of ECs, and here we showed that HBMSCs cultured at high density effectively took up LDL, implying an EC-like state. However, we cannot exclude that the

increased LDL uptake might, in part, reflect fat accumulation. When the cells were plated on Matrigel or on CAM membranes, it was clear that they not only produced much larger tubules but also enhanced the expression of several EC markers. Taken together, these data indicate that the high density cells were committed to the EC lineage.

Our experiments indicate that low density culture suppresses the potential of HBMSCs to differentiate along vascular lineages. Therefore, a double-labeling immunofluorescence approach was used to address whether our HBMSC populations might be bipotential with the ability to differentiate along either SMC or EC lineages. Many cells were found to express both vSMC and EC markers, so it was clear that both lineages can be induced in the same cells over an initial 24 h after plating at high density. However, there may be some heterogeneous potential within our HBMSC populations.

HBMSCs are known to have therapeutic potential for tissue remodeling and repair. Being multipotent, they can not only differentiate along a range of lineages, they can also enhance tissue repair by secreting anti-inflammatory factors (Lee et al., 2009b). Their culture conditions can affect their phenotypic status; for example, culture in 3D spheres was recently shown to enhance the expression of anti-inflammatory molecules (Bartosh et al., 2010). In this study, we have demonstrated that it is possible to control their vascular differentiation *in vitro*, through regulating plating density and cell–cell contact. High density triggers EC differentiation by rapid induction of Notch signaling which in turn up-regulates VEGF-A, a growth factor that consolidates EC differentiation. These high density *in vitro* conditions may mirror extensive cell contact areas in EC monolayers *in vivo*. Our study, which has defined mechanisms of vascular differentiation of HBMSCs, provides new opportunities to regulate HBMSCs in therapeutic neovascularisation.

## Conclusion

This study has highlighted the coordinated role of cell density-dependent Notch signaling and Notch-induced VEGF-A in directing the vascular fate of HBMSCs.

## Materials and methods

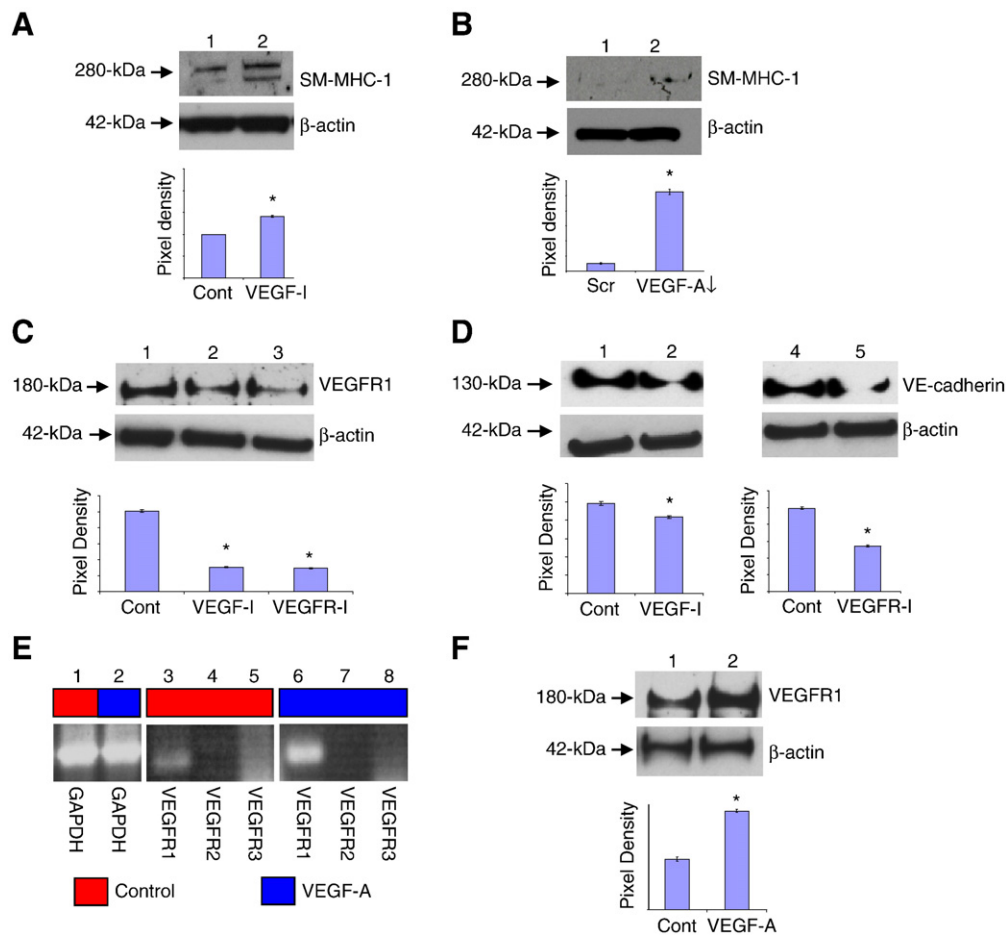
### Antibodies

Primary antibodies used for immunoblot and immunofluorescence analysis were goat anti-human VEGFR1 (AF321), rabbit anti-human phospho-VEGFR1 (Y1213) (AF4170), and goat anti-human hairy enhancer of split-1 (HES-1) (AF3317), all from R&D Systems. Mouse anti-human von Willebrand factor (vWF) (3H3126) (sc-73267), mouse anti-human vascular cell adhesion molecule-1 (VCAM-1) (P3C4) (sc-20070), mouse anti-human phosphotyrosine (PY99) (sc-7020), rabbit anti-human smoothelin B (sc-28562), rabbit anti-human Notch 3 (m-134) (sc-5593), and goat anti-human Notch 4 (E-12) (sc-32613) were all from Santa-Cruz Biotechnology. Rabbit anti-human Notch 1 (C37C7), rabbit anti-human Notch 2 (8A1), mouse anti-human platelet/endothelial cell adhesion molecule-1 (PECAM-1) (89C2) (3528), rabbit anti-human vascular endothelial cadherin (VE-cadherin) (2158) and rabbit anti-human VEGFR2 (55B11) (2479) were all from Cell Signaling Technology. Mouse anti-human smooth muscle alpha actin (αSMA) (clone 1A4), mouse anti-human calponin (clone Calp), mouse anti-human smooth muscle myosin heavy chain 1 (SM-MHC-1) (clone SMMS-1), and negative control mouse IgG<sub>1</sub> (X093101) were purchased from DAKO.

### Growth factors and inhibitors

VEGF-A<sub>165</sub> (298-VS) and VEGF neutralising monoclonal antibody (clone 26503) were obtained from R&D Systems. VEGFR tyrosine





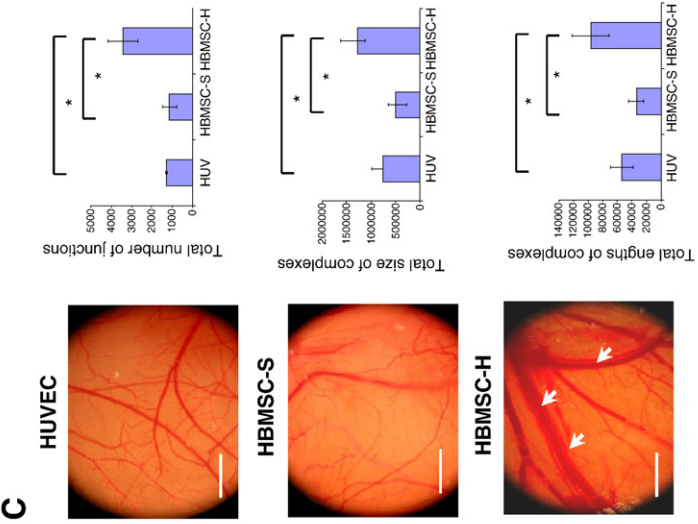
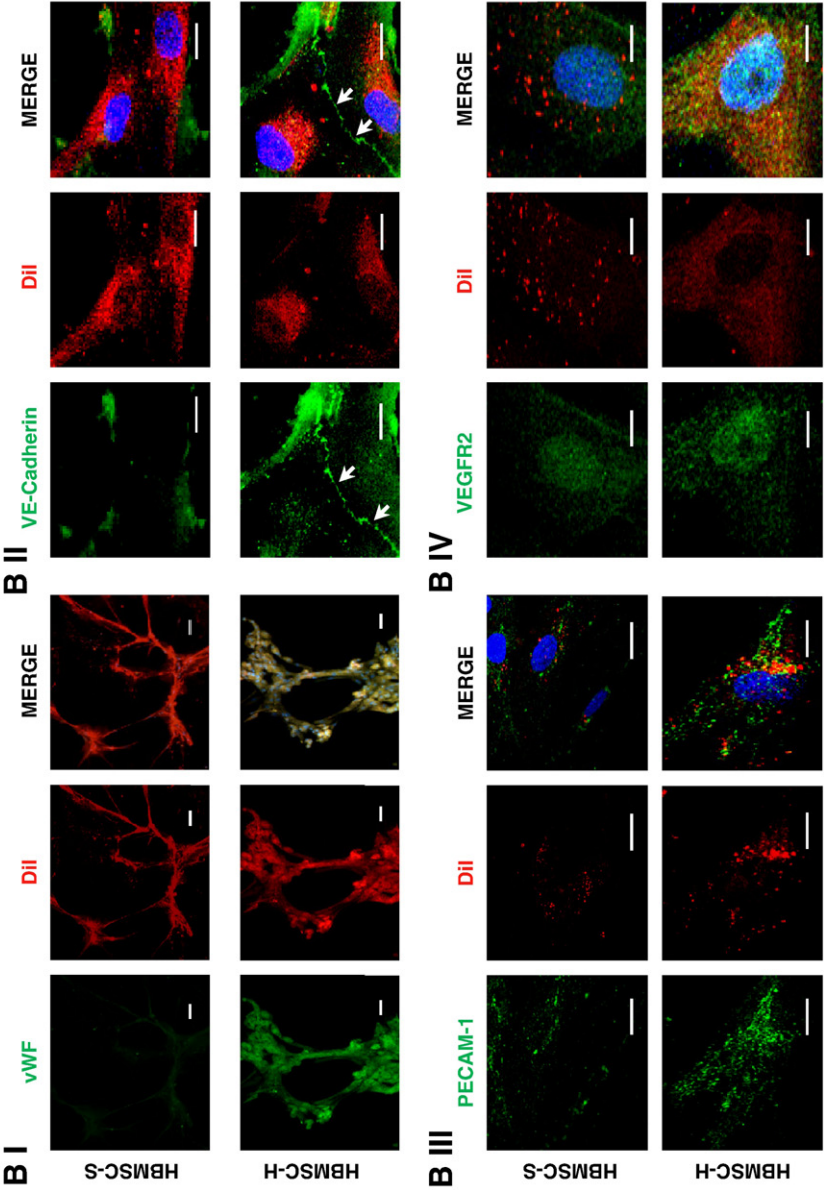
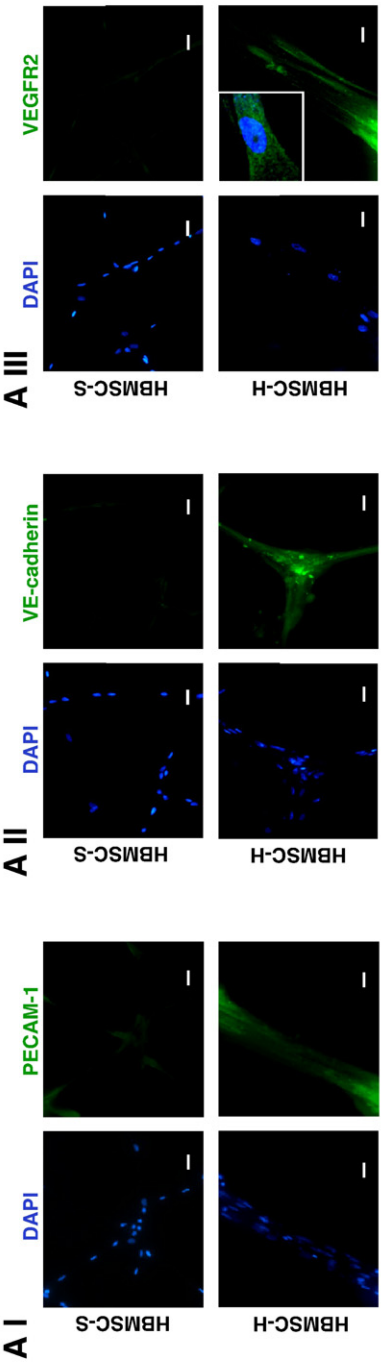
**Figure 6** VEGF-A inhibited HBMSC differentiation to vSMCs but promoted EC differentiation. To determine whether secreted VEGF-A inhibited vSMC marker expression in HBMSCs cultured at high density, the effects of blocking VEGF-A signaling was determined. (A,B) HBMSCs cultured at high density for 24 h were (A) exposed to 1  $\mu$ g/ml VEGF neutralisation antibody (VEGF-I) (lane 2) or (B) transfected with VEGF-A siRNA (lane 2), and then SM-MHC-1 expression was determined by immunoblot analysis. Untreated HBMSCs (Cont) or scrambled (Scr) siRNA-transfected HBMSCs (lane 1) were used as respective controls. Pixel density was normalised to  $\beta$ -actin and plotted as a bar graph. \*  $P < 0.05$  compared to controls. Data are representative of two independent experiments for each analysis. (C,D) HBMSCs cultured at high density for 14 days were exposed to 1  $\mu$ g/ml VEGF-I (lane 2) or 0.5  $\mu$ M VEGFR tyrosine kinase inhibitor (VEGFR-I) (C, lane 3, and D, lane 5), and then (C) VEGFR1 and (D) VE-cadherin expression was determined by immunoblot analysis. Untreated HBMSCs were used as controls (Cont) (C, lane 1, and D, lanes 1 and 4). Pixel density was normalised to  $\beta$ -actin and plotted as a bar graph. \*  $P < 0.05$  compared to untreated HBMSCs. A representative of two independent experiments is shown in each case. (E) HBMSCs cultured at high density for 14 days were exposed to 50 ng/ml recombinant VEGF-A, and then VEGFR transcript expression was determined by semiquantitative RT-PCR analysis. Lanes 1–2, GAPDH in untreated (control) HBMSCs. VEGFRs 1–3 in untreated (control) HBMSCs, lanes 3–5, or following exposure to recombinant VEGF-A, lanes 6–8, respectively. Two different primer pairs for VEGFRs 1–3 gave similar results. Red boxes denote unstimulated cells; blue boxes denote VEGF-A-stimulated cells. (F) Immunoblot analysis of VEGFR1 protein level in untreated HBMSCs (lane 1), or following exposure to recombinant VEGF-A for 14 days (lane 2). \*  $P < 0.05$  compared to untreated HBMSCs cultured after plating at high cell density (HBMSC-H). In each case, fresh medium supplemented with VEGF-A or inhibitors was added to cells every 2 days.

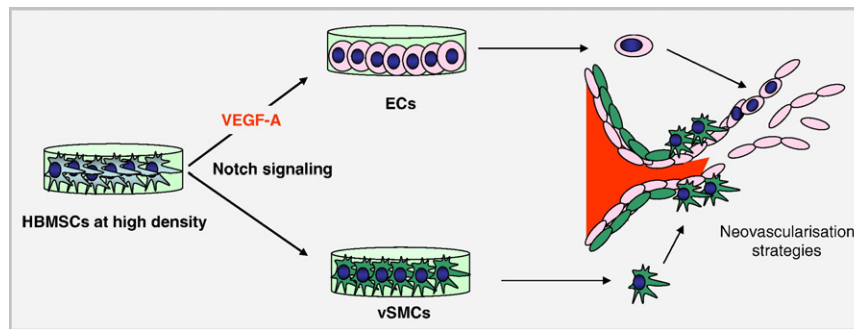
kinase inhibitor (KRN633) was purchased from Calbiochem. The gamma secretase inhibitor *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-5-phenylglycine *t*-butyl ester (DAPT) was obtained from Sigma-Aldrich.

## Cell culture

Human bone marrow stromal cells were obtained from the bone marrow of a 28-year-old female and from a 21-year-old male (Lonza). The HBMSCs had a typical spindle-shaped morphology (Supplementary

Fig. S1A), were positive for CD73, CD29, CD44, CD51, and CD105, but negative for the haematopoietic cell marker CD34 (Supplementary Fig. S1B), and had the potential to differentiate toward adipocyte, osteocyte, or chondrocyte lineages (Supplementary Fig. S1C). HBMSCs cultured on 0.1% gelatin (Sigma-Aldrich) were maintained in basal medium (Invitrogen) supplemented with 2% L-glutamine and 0.1% penicillin/streptomycin. HBMSCs were cultured at 37 °C in a humidified atmosphere of 20% O<sub>2</sub>, 5% CO<sub>2</sub>, and used at passage 4–5. Human umbilical vein endothelial cells from 43-year-old and 29-year-old females (Invitrogen) were maintained in endothelial cell basal medium (Lonza), containing singleQuot supplements, and used at passage 4.





**Figure 8** Model showing how Notch signaling and VEGF-A direct the vascular differentiation of cultured HBMSCs. HBMSCs plated and cultured at high density are induced to differentiate to an EC fate through Notch signaling and Notch-induced VEGF-A. These mechanisms may be therapeutically applicable for neovascularisation.

Human dermal fibroblasts (HDFs) from a 51-year-old male, obtained from the European Collection of Cell Cultures, were maintained in Dulbecco's minimal essential medium (DMEM) (Invitrogen) containing 10% fetal bovine serum, 1% L-glutamine, 0.2% penicillin/ streptomycin, and used at passage 8.

### Cell plating

For standard culture conditions, cells were seeded at 70 000 cells in 9.6 cm<sup>2</sup> at initial plating (corresponding to 70% confluence). For high cell density experiments, HBMSCs were seeded at 100 000 cells in 9.6 cm<sup>2</sup> at initial plating. For low density experiments, HBMSCs were seeded at 10 000 cells in 9.6 cm<sup>2</sup> at initial plating.

### Semiquantitative and quantitative reverse-transcription polymerase chain reaction

Total RNA was isolated from cultured cells using Trizol reagent. Total RNA (1 µg) was reverse-transcribed and subjected to polymerase chain reaction (PCR). Quantitative PCR was performed using a SYBR green quantitative PCR core kit (Eurogentec), with each sample run in triplicate. Data were normalized to GAPDH, and relative expression was calculated according to the 2<sup>-[delta]CT</sup> formula. Oligonucleotide primers for PCR were designed using Primer3 software. Each primer pair was designed using the same parameters (70- to 100-bp product size and *T<sub>m</sub>* of 60 °C). PCR were repeated using a second primer set (sequences not shown). Reaction products were resolved using 2.5% ultrapure agarose (Invitrogen) gel

electrophoresis run at 100 V for 2 h in 1X TAE (Tris base, acetic acid, EDTA) buffer containing 2.5 µl Gel Red (Biotium), and visualised using a transilluminator.

### Primer sequences

Primer sequences were as follows: GAPDH (71 bp), forward (5'-AAGGGCATCCTGGGCTAC-3') and reverse (5'-GTGGAGGAGTGGGTGTCG-3'); VEGFR1 (99 bp), forward (5'-GCGACGTGTGGTCTTACG-3') and reverse (5'-GGCGACTGCAAAAGTCCT-3'); VEGFR-2 (81 bp), forward (5'-CATCCAGTGGGCTGATGA-3') and reverse (5'-TGCCACTTCCAAAAGCAA-3'); VEGFR-3 (87 bp), forward (5'-GATGCGGGACCGTATCTG-3') and reverse (5'-ATCCTCGGAGCCTTCCAC-3'); HES1 (91 bp), forward (5'-CCAAAGACAGCATCTGAGCA-3') and reverse (5'-TCAGCTGGCTCAGACTTTCA-3'); VEGF-A (98 bp), forward (5'-CACCCATGGCAGAAGGAG-3') and reverse (5'-CACCAGGGTCTCGATTGG-3'); VE-cadherin (74 bp), forward (5'-GGAGCCGAGCATGTGTCT-3') and reverse (5'-TCTGCAAGGTGTGCCTGA-3').

### Immunoblot analysis

Cells were washed with phosphate-buffered saline (PBS), incubated with lysis buffer for 15 min on ice, and then isolated. Total protein lysates were quantitated using a bicinchoninic acid (BCA) assay (Pierce), electrophoresed under reducing conditions using NuPAGE 4–12% Bis-Tris gels at 200 V for 2 h, and then transferred to a nitrocellulose membrane using a NuPAGE Western transfer system (Invitrogen). Membranes were blocked in 4% milk solids and then

**Figure 7** Expression of endothelial markers by high density HBMSCs on Matrigel and chick chorioallantoic (CAM) membranes. (A) To study the capacity of HBMSCs to form tubules, 20 000 HBMSCs cultured at high density (HBMSC-H) for 28 days, or cultured under standard conditions (HBMSC-S) for 7 days, were seeded onto growth factor reduced Matrigel in 0.5% serum DMEM for 48 h. (A I–III) Immunofluorescence analysis of (A I) PECAM-1, (A II) VE-cadherin, and (A III) VEGFR2; inset depicts an enlarged region of the image. All immunofluorescent images were taken using an Olympus BX51 widefield microscope (20x objective). Scale bars represent 20 µm. A representative of two independent experiments is shown in each case. (B) The effect of exposing HBMSCs to the angiogenic environment of the chick chorioallantoic membrane (CAM) was investigated. To identify implanted cells from those of the chick, 20 000 HBMSCs were prelabeled with Dil, seeded onto Matrigel-treated coverslips, and then placed in direct contact with the CAM of a Day 6 chick embryo for 48 h. (B I) Immunofluorescence analysis of vWF, using an Olympus BX51 widefield microscope (20x objective). vWF, green; Dil, red. Scale bars represent 100 µm. (B II–IV) Immunofluorescence analysis of (II) VE-cadherin, (III) PECAM-1, and (IV) VEGFR2, using a Nikon C1 upright confocal microscope (60x objective). White arrows indicate that VE-cadherin is present at the cell surface. Scale bars represent 20 µm. VE-cadherin, PECAM-1, or VEGFR2, green; Dil, red. (C) CAM vascularisation following exposure to HUVECs or HBMSC-coated coverslips for 48 h. White arrows indicate where the presence of HBMSCs precultured at high density (HBMSC-H) induced larger blood vessels in the CAM. The degree of vascularisation was quantified with Angioquant software measuring total number of junctions, total size of complexes, and total lengths of complexes. \* *P* < 0.05. Ten representative images were taken for each treatment, using a Nikon stereomicroscope with each analysis performed in triplicate. Scale bars represent 6 mm. A representative of two independent experiments is shown in each case.

incubated with the primary antibody overnight at 4 °C (1:500–1:1000 dilution). Membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (DAKO, UK) for 2 h and then developed with SuperSignal West Dura Extended Duration Chemiluminescence (Thermo Fisher Scientific). Proteins were visualised using KODAK X-AR film and density of bands was determined using Gene Tools software (Syngene), and then the corresponding loading control was normalised.

### Immunofluorescence microscopy

After culturing cells on gelatin-coated 1.7 cm<sup>2</sup> coverslips, cells were washed with PBS and fixed in 4% paraformaldehyde for 20 min, quenched in 0.2 M glycine (Fisher Scientific) for 20 min, and then permeabilised in 0.5% Triton X-100 (Sigma-Aldrich) for 4 min. Nonspecific binding was blocked with 2% fish skin gelatin solution (Sigma-Aldrich) for 1 h, and then cells were incubated overnight at 4 °C with primary antibodies (1:50–1:100 dilution) in blocking solution. After washing with PBS, cells were incubated with Alexafluor secondary antibodies (Alexa-Fluor 488 conjugated or Alexa-Fluor 546/555 conjugated (Invitrogen) (1:200 dilution) for 2 h at room temperature. For F-actin staining, cells were also incubated with rhodamine-conjugated phalloidin (Invitrogen) (1:500 dilution) for 2 h at room temperature. Cells were then washed and coverslips mounted using Prolong Gold Antifade solution containing 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen). At least 20 representative images of each sample were captured for each analysis. All immunofluorescence experiments included no primary antibody, no secondary antibody, and isotype-specific IgG<sub>1</sub> controls to confirm specificity (data not shown).

For fluorescence microscopy, images were collected on an Olympus Widefield BX51 upright microscope using a 20x/UPlanFLN objective and captured using a Coolsnap HQ camera (Photometrics) through MetaVue Software (Molecular Devices). Specific band pass filter sets for DAPI, FITC, and tetramethylrhodamineisothiocyanate (TRITC) were used to prevent bleed-through from one channel to the next. For confocal microscopy, images were collected using a Nikon C1 confocal on an upright 90i microscope with a 60x/1.40 Plan Apo objective and 3x confocal zoom. Images for DAPI, FITC, and Texas red were excited with the 405, 488, and 543 nm laser lines, respectively. Images were then processed and analysed using Nikon EZ-C1 FreeViewer v3.3 software. For Delta Vision microscopy, a Delta Vision restoration microscope (Applied Precision) 40x/0.85 UPlan Apo objective and Coolsnap (Photometrics) camera was utilised. Raw images were deconvolved using the Softworx software; maximum intensity projections of these deconvolved images are shown.

### Flow cytometry

Following trypsinisation, cells were left to recover in growth medium for 1 h, washed twice in wash buffer (0.5% bovine serum albumin in PBS) and 1×10<sup>5</sup> cells incubated with either phycoerythrin (PE) conjugated or unconjugated primary antibody for 1 h, and then washed three times with wash buffer. For an unconjugated antibody, cells were additionally incubated with a species-specific fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:200) (Dako) for 45 min and then washed three times with wash buffer. For each analysis, 100 000 cells were counted using a FACscan cytometer at a flow rate less than 200 events per second.

### VEGF-A detection

Enzyme-linked immunosorbent (ELISA) assays were performed using the Quantikine human VEGF-A immunoassay (R&D Systems), according to the manufacturer's protocol.

### Notch activation

Notch was activated either by ethylenediaminetetraacetic acid (EDTA) (Rand et al., 2000) or recombinant Jagged-1. For EDTA experiments, cells were stimulated for 15 min with 5 mM EDTA, washed twice with basal medium, and left to recover for 24 h, before isolation. For Notch activation using Jagged-1, recombinant human Jagged-1/Fc chimera (1277-JG) (R&D Systems) was immobilised on culture plates by incubating plates with a solution of Jagged-1 (5 µg/ml) in PBS for 2 h at 37 °C. Control plates did not contain recombinant human Jagged-1. For these experiments, HBMSCs were seeded onto plates at low or high density and cultured for 24 h.

### HBMSC differentiation assay

HBMSCs were induced to differentiate toward adipocyte, osteocyte, or chondrocyte lineages using an MSC functional identification kit (R&D Systems), according to the manufacturer's protocol, and then cells were prepared for immunofluorescence analysis as previously described using the antibodies provided. In addition, lipid droplets were stained using boron-dipyrromethene (Bodipy) 493/503 (Invitrogen) and F-actin filaments stained using phalloidin.

### siRNA knockdown transfections

HBMSCs (5×10<sup>5</sup> cells), together with 3 µg small interfering RNAs (siRNAs), were transfected by electroporation using a human Nucleofector kit (Lonza), and cultured overnight in growth medium. Validated siRNAs, which were functionally tested to provide ≥70% target gene knockdown, were used for VEGF-A knockdown (Qiagen). For Notch knockdowns, siRNAs for Notch1 (sc-36095), Notch2 (sc-40135), and Notch3 (sc-37135) (Santa Cruz, USA) were employed, with scrambled siRNA as a control (Qiagen).

### Immunoprecipitation analysis

Cell lysates were precleared using 10% (w/v) protein A-Sepharose (GE Healthcare), and then precleared lysates were incubated with primary antibody overnight at 4 °C. Immune complexes were isolated by incubation with 10% (w/v) protein A-Sepharose for 2 h at 4 °C. Protein A-Sepharose beads were washed three times using ice-cold lysis buffer, and then resuspended in NuPAGE gel loading buffer (Invitrogen).

### Endothelial functional tests

#### LDL uptake assay

ECs readily take up 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine-labeled acetylated low density lipoprotein (Dil-Ac-LDL), where the lipoprotein is degraded by lysosomes and fluorescent Dil accumulates within intracellular membranes. Cells were incubated with 10 µg/ml Dil-Ac-LDL (BT-906) (Biomedical Technologies Ltd) for 3 h at 37 °C, washed with PBS, fixed in 4% paraformaldehyde for 20 min, and then quenched in 0.2 M glycine for 20 min. Uptake of Dil-Ac-LDL was assessed by mounting coverslips using Prolong Gold Antifade solution containing DAPI (Invitrogen) and examination using an Olympus Widefield BX51 microscope.

#### Induction of vascular cell adhesion molecule-1 in response to TNFα

Recombinant human tumour necrosis factor α (TNFα) (10 ng/ml) (R&D Systems; 210-TA-010) was added to cells for 24 h, and then cells were prepared for immunofluorescence analysis as previously described.



### Matrigel tubule formation assay

A thin layer of growth factor-reduced Matrigel (BD Biosciences) was used to coat round glass coverslips and allowed to set for 30 min at 37 °C. Cells (20 000) in 0.5% serum containing DMEM were seeded onto the surface of Matrigel and incubated at 37 °C for up to 48 h to allow tubule formation, and then prepared for immunofluorescence analysis as previously described.

### Chorioallantoic membrane (CAM) angiogenesis assay

Fertilized white chicken eggs were incubated for 6 days at 37.5 °C and 43% humidity in a specialised egg incubator. To distinguish implanted cells from chick cells on the CAM, implanted cells were prelabeled with 10 µg/ml 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate Dil (Sigma-Aldrich) for 30 min at 37 °C, seeded onto Matrigel-coated coverslips, and then left to adhere for 1 h. Eggs were cleaned with 70% ethanol and a square window was cut into the shell with dissecting scissors to reveal the underlying embryo and CAM blood vessels. The membrane covering the CAM surface was carefully removed with forceps and coverslips were implanted cell face down onto the CAM. The window was sealed with transparent tape and the egg was returned to the incubator for 48 h at 37.5 °C and 65% humidity, after which coverslips were removed and prepared for immunofluorescence as previously described.

### Statistical analysis

In all quantitation experiments, results were expressed as the mean standard deviation. Statistical differences between sets of data were determined using a paired *t* test on SigmaPlot 8.0 software, with *P* < 0.05 considered significant.

### Acknowledgments

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### Appendix A. Supplementary data

Supplementary materials related to this article can be found online at [doi:10.1016/j.jscr.2011.02.001](https://doi.org/10.1016/j.jscr.2011.02.001).

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